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Metabolic Effect of 3,3',5'-Triiodothyronine in Cultured Growth Hormone-producing Rat Pituitary Tumor Cells

Evidence for a Unique Mechanism of Thyroid Hormone Action

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Abstract

Physiologic levels of 3,3',5'-triiodothyronine (rT_3) are generally believed to have minimal metabolic effects in the pituitary gland and other tissues. In the present studies, the regulatory role of rT_3 and other thyroid hormones on iodothyronine 5'-deiodinase (I5'D) activity was studied in a growth hormone-producing rat pituitary tumor cell line (GH_3 cells). I5'D activity was thiol-dependent and displayed nonlinear reaction kinetics suggesting the presence of two enzymatic processes, one having a low Michaelis constant (K_m for thyroxine [T_4] of 2 nM) and a second with a high K_m value (0.9 μM). Growth of cells in hormone-depleted medium resulted in a two- to 3.5-fold increase in low K_m I5'D activity ($P < 0.001$). The addition of thyroid hormones to the culture medium resulted in a rapid, dose-dependent inhibition of low K_m I5'D activity with the following order of analogue potency: $rT_3 \geq T_4 > $ 3,5,3'-triiodothyronine (T_3). Using serum-free culture conditions, rT_3 was ~50 times more active than T_3. These inhibitory effects were noted within 15 min of hormone addition and could not be attributed to substrate competition with T_4. These findings suggest that the control of T_4 to T_3 conversion by thyroid hormones in the anterior pituitary gland is mediated by a unique cellular mechanism that is independent of the nuclear T_3 receptor; and under some circumstances, rT_3 may play a regulatory role in controlling this enzymatic process.

Introduction

The metabolic actions of thyroid hormones appear to be mediated primarily through the interaction of 3,5,3'-triiodothyronine (T_3)[1] with specific nuclear receptors (1). In the various growth hormone-producing rat pituitary tumor cell lines (GH cells) for example, T_3 is the most potent thyroid hormone analogue in stimulating glucose consumption (2), growth hormone production (3), and amino acid transport (4), and these effects correlate well with the proportion of nuclear binding sites occupied by T_3. Although 3,3',5'-triiodothyronine (rT_3) also has agonist activity in GH cells, 1,000-fold greater concentrations are required, presumably due to the nuclear receptor having a significantly lower affinity for rT_3 (5). In other systems, the metabolic effects of rT_3 also appear to be minimal (5).

In the present studies, we used the GH_3 cell line to investigate the regulatory effects of thyroid hormones on a low Michaelis constant (K_m) iodothyronine 5'-deiodinase (I5'D) process (type II). Our finding that rT_3 is a potent and rapid inhibitor of this enzymatic process suggests that thyroid hormone control of T_3 formation in anterior pituitary tissue is mediated through a unique cellular mechanism that is independent of the nuclear T_3 receptor.

Methods

GH_3 cells were obtained from the American Type Culture Collection (Rockville, MD) and were routinely grown in 75-cm^2 plastic flasks and 100-mm plastic culture dishes using Ham's F-10 culture medium supplemented with 2.5% fetal calf serum (FCS), 15% horse serum (HS), and gentamycin (50 μg/ml) in a humidified atmosphere of 5% CO_2/95% air. Medium was routinely changed twice weekly. In some experiments cells were grown to near confluence in standard medium and then transferred and maintained for 4 d in serum-free Ham's F-10 or in Ham's F-10 supplemented with FCS (10%) that previously had been treated with either charcoal (6) or an ion exchange resin (7). According to the manufacturer's (HyClone Laboratories, Logan, UT) specifications, the T_4 and T_3 levels were 12.7 μg/dl and 118 ng/dl, respectively, in the FCS and 2.9 μg/dl and 47 ng/dl, respectively, in the HS. Treatment of FCS either with charcoal or with an ion exchange resin was demonstrated to remove >99% of the iodothyronines.

Experiments were performed with cells at the near confluent stage of growth and 48 h after the last medium change. Cells were harvested by scraping with a rubber policeman, washed twice with ice-cold buffer (0.25 M sucrose, 0.02 M Tris-HCl, pH 7.6, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM dithiothreitol [DTT], 5% glycerol), then resuspended in the same buffer and sonicated using a sonic dismembrator (Artek Systems Corp., Farmingdale, NY) for 25 s at a setting of 45. The sonicate was centrifuged at 800 *g* for 10 min and the supernatant was used immediately for determination of I5'D activity as previously described (8, 9). T_3 production rates were quantified by radioimmunoassay (RIA) using stable thyroxine (T_4) as a substrate at concentrations of 0.002–3.0 μM in the presence of added DTT. The T_4 content of the supernatant fraction, before the addition of stable T_4 or enrichment with DTT, was determined by RIA in ethanol extracts (8). Protein concentration was determined by the method of Lowry et al. (10).

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1. *Abbreviations used in this paper:* DTT, dithiothreitol; FCS, fetal calf serum; GH cells, growth hormone-producing cell lines; HS, horse serum; I5'D, iodothyronine 5'-deiodinase; K_m, Michaelis constant; rT_3, 3,3',5'-triiodothyronine; T_3, 3,5,3'-triiodothyronine; T_4, thyroxine.

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Several experiments were performed to characterize and define the optimal conditions for quantifying I5′D activity in GH_3 cells: (*a*) enzyme activity in supernatants was found to be a linear function of both protein concentration (up to 11 mg/ml) and incubation time (up to 45 min) and was dependent on the presence of sulfhydryl reducing agents (maximum activity at 20 mM DTT). (*b*) No T_3 formation was noted in supernatants heated to 80°C for 45 min before incubation with T_4 and DTT. (*c*) Incubation of supernatants with known concentrations of stable T_3 (at 37°C for 45 min in the presence of 20 mM DTT) failed to disclose any evidence of T_3 degradation. (*d*) Enzyme activity determined 1–4 d after a medium change varied by <15% and was the same during either the log or near confluent stages of growth.

The uptake of rT_3 by GH_3 cells was determined by incubating cells in medium containing a known specific activity of ^{125}I-rT_3 (New England Nuclear, Boston, MA). Cells were then harvested, washed, and sonicated as above, and the amount of radioactivity in the supernate fraction was determined. The rT_3 content was calculated after correcting for the proportion of counts due to ^{125}I as determined by electrophoresis (11). Stable rT_3 was obtained from Calbiochem-Behring Corp., La Jolla, CA.

Kinetic data were analyzed using Eadie-Hofstee plots, and nonlinear plots were resolved into two components as previously described (9). Statistical comparison was performed using the unpaired *t* test. All results are given as mean±SE.

Results

I5′D activity in GH_3 cell supernatants demonstrated nonlinear reaction kinetics suggesting the presence of two enzymatic processes having K_m values of 2.4±1.0 nM (n = 4 experiments) and 0.9±0.3 μM (Fig. 1). Corresponding values for the maximum velocity were 23±7 fmol T_3/min mg protein and 32±6 fmol T_3/min mg protein. The addition of 0.5 mM 6-*n*-propyl-2-thiouracil to cell sonicates (in the presence of 5 mM DTT) inhibited T_3 production by <10% at low substrate concentrations (10 nM T_4) but by 53% ($P < 0.001$) at T_4 concentrations of 3 μM (data not shown). In subsequent experiments I5′D activity was determined using supernatant substrate concentrations of 10 or 50 nM T_4 and 20 mM DTT; these conditions permit quantification of primarily the low K_m process.

The effect on I5′D activity of culturing cells in medium supplemented with different serum preparations is shown in Fig. 2. Cells grown in Ham's F-10 medium supplemented with 10% FCS manifested only 46% of the activity noted in cells cultured in standard medium. However, cells maintained in serum-free medium or in the presence of 10% resin-treated or charcoal-treated FCS showed a two- to 3.5-fold increase in I5′D activity, suggesting that one or more components of serum exerted inhibitory effects on enzyme activity. The addition of T_4, T_3, or rT_3 to the culture medium resulted in significant inhibitory effects on I5′D activity. Dose response curves, as determined in serum-free medium, are shown in Fig. 3. In this experiment, rT_3 was somewhat more active than T_4 and 50 times more active than T_3 in inhibiting I5′D activity. Additional experiments (n = 5) using both serum-free conditions and medium supplemented with resin-treated FCS confirmed that the order of potency of these analogues was $rT_3 \geq T_4 > T_3$. In medium supplemented with resin-treated FCS, T_3 concentrations of $>10^{-9}$ M were required to inhibit I5′D activity. Thyronine, at concentrations up to 10^{-7} M, inhibited this process by <10%.

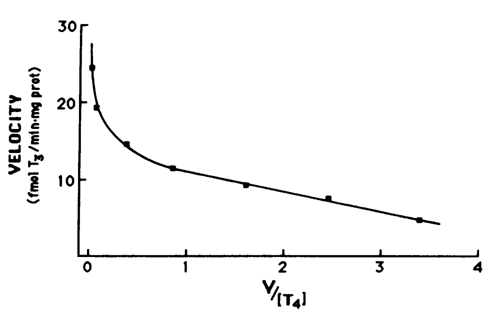

Figure 1. Kinetic analysis, utilizing an Eadie-Hofstee plot, of I5′D activity in GH_3 cells. Cells were cultured in standard medium containing 2.5% FCS and 15% HS. Enzyme activity was determined using T_4 concentrations of 0.002–1.0 μM and 20 mM DTT. T_4 concentrations on the abscissa are expressed in nanomoles per liter. Points represent the mean of duplicate values, which differed <15%. Prot, protein.

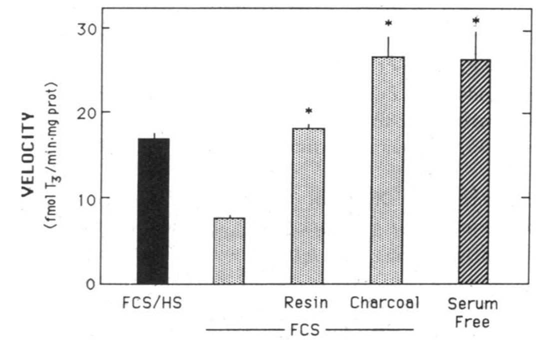

Figure 2. The effect of different medium conditions on I5′D activity in GH_3 cells. Cells were grown to near confluence in standard medium (FCS/HS) and then transferred and maintained for four additional days in the medium indicated. I5′D activity was determined using a T_4 concentration of 10 nM and 20 mM DTT. Values represent the mean±SE of triplicate determinations. •, $P < 0.001$ compared with cells maintained in untreated FCS. Prot, protein.

The potential of rT_3 acting as a competitive substrate with T_4 in the reaction mixture was assessed by determining the rate of T_3 formation in the presence of both added T_4 (50 nM) and rT_3. Concentrations of rT_3 of up to 10 nM had no effect on T_3 production rates. Quantitation of rT_3 cellular uptake revealed that 4 h after the addition of 10^{-8} M rT_3 to the culture medium, supernatant concentrations of rT_3 were ~5 nM in cells grown in serum-free medium and 0.4 nM in cells maintained in medium supplemented with resin-treated FCS. Thus, even at high medium rT_3 concentrations, the supernatant concentrations of rT_3 were insufficient to affect T_3 production rates when 50 nM T_4 was provided as substrate in the reaction mixture. Furthermore, the concentration of T_4 (as determined by RIA) in supernatants derived from cells grown in medium supplemented with resin-treated FCS and either 10^{-8} M or 10^{-7} M T_4 were 0.7 nM and 7 nM, respectively; concentrations that would not add significantly to the rates of T_3 formation in the reaction mixture.[2]

2. Based on the measured sonicate protein concentration and the known values of protein content and cellular volume of GH cells (12), the actual intracellular concentrations of rT_3 and T_4 can be calculated. For cells incubated for 4 h in medium containing 10% FCS, intracellular rT_3 and T_4 concentrations were equal to the total medium concentrations of these iodothyronines. Under serum-free medium conditions, the intracellular concentrations were ~30-fold greater than the medium concentrations.

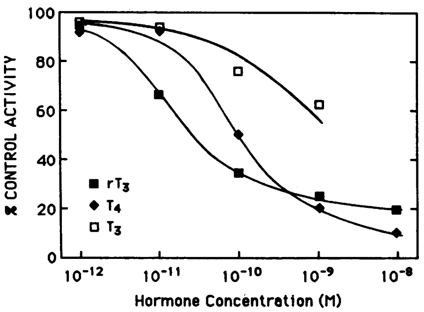

Figure 3. Dose response curves of the inhibitory effects of thyroid hormones on I5'D activity in GH_3 cells. Cells were grown to near confluence in standard medium and then transferred and maintained for four additional days in serum-free Ham's F-10 medium. Iodothyronines were added to the culture medium 4 h before harvesting. I5'D activity was determined using a T_4 concentration of 50 nM and 20 mM DTT. Points represent the mean of triplicate determinations, which differed <5%.

The regulatory effect of rT_3 on I5'D activity was shown to be quite rapid (Fig. 4). Significant inhibition was noted within 15 min of adding rT_3 to the medium and was near maximal at 2 h. A similar time course of inhibition was shown for T_4.

Discussion

I5'D activity in GH_3 cells was shown in the present studies to be a thiol-dependent process with nonlinear reaction kinetics suggesting the presence of two enzymatic pathways having markedly different K_m values for T_4. A differential sensitivity of these two processes to the inhibitory effects of PTU was also noted. These findings are similar to those previously described in homogenates of rat anterior pituitary glands (9, 13) and cerebral cortex (14). Our findings differ, however, from a prior report using GH_3 cells where only a high K_m process (3.8 μM) was identified (15). The failure to detect a low K_m process in this latter study may have been secondary to the low concentrations of DTT used (2 mM) and the relative insensitivity of the T_3 RIA (3.75 pg/tube lower limits of detection vs. 0.2 pg/tube in the present studies [8, 9]).

Recent studies have shown that low K_m I5'D activity is responsible for the vast majority of "local" T_3 formation in the pituitary and depends on thyroid hormone status (8, 13). In hypothyroid animals, the activity of this process is markedly elevated, whereas thyroid hormone administration induces a rapid decrease in T_4 to T_3 conversion. Our finding that low K_m I5'D activity in GH_3 cells rapidly responds to medium thyroid hormone concentrations supports the use of this cell line as a model system.

The effects of thyroid hormones on I5'D activity in various tissues are complex (16). In liver and kidney homogenates, rT_3 inhibits T_3 formation by substrate competition with T_4 for a high K_m (type I) I5'D process (17). The suppressive effects of thyroid hormones on low K_m (type II) I5'D activity in GH_3 cells, however, cannot be attributed to this mechanism. Our demonstration that rT_3 is a potent inhibitor of this process contrasts sharply with the weak agonist activity of this analogue on other metabolic processes (2, 5) and strongly suggests that a unique regulatory mechanism, independent of the nuclear T_3 receptor, is responsible for this effect. Further evidence for this is the finding that relatively high concentrations of T_3 (10^{-9} M in medium containing serum), which have been shown by other investigators to stimulate significantly growth hormone production and maximally suppress thyrotropin-releasing hormone receptors in GH_3 cells (18), had no effect on I5'D activity. A recent report (19) demonstrating that the injection of T_4 or rT_3 in the rat is more effective than T_3 in inhibiting pituitary and cerebral cortex I5'D activity, suggests that the order of iodothyronine potency found in GH_3 cells is also applicable in vivo. The cellular processes responsible for these regulatory effects are uncertain. Leonard et al. (20), however, have recently suggested that the T_3-dependent inhibition of I5'D activity in rat pituitary and cerebral cortex is mediated by a posttranscriptional mechanism that increases the rate of degradation or inactivation of the enzyme.

To date, a 5-deiodinase process has not been described in pituitary tissue and none could be demonstrated in the present study in GH_3 cells when T_3 was used as a substrate. This finding, together with the near equal potency of T_4 with rT_3, suggests that the regulatory effects of T_4 on I5'D activity are direct and not mediated by conversion to rT_3.

An intriguing question raised by these studies is the possible role of rT_3 in the control of T_4 to T_3 conversion in the pituitary and brain. Although rT_3 appears to be a potent regulator of this metabolic process, its free concentration in normal human serum is ~40-fold lower than that of T_4 (21). Thus, T_4 rather than rT_3 is likely to exert the principle physiologic control over this process. This thesis is consistent with reports that the administration of rT_3 to normal human subjects has no effect on the serum levels of T_4, T_3, thyrotropin, or the thyrotropin response to thyrotropin-releasing hormone (22, 23). In nonthyroidal illness, however, marked alterations occur in thyroid hormone metabolism, which may result in decreased serum free T_4 levels and increases in serum free rT_3 levels of up to 15-fold (21). Under such circumstances, inhibitory effects of rT_3 on I5'D activity may become significant.

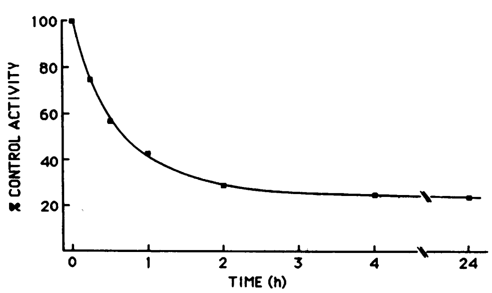

Figure 4. Time course of the inhibitory effect of 10^{-8} M rT_3 on I5'D activity in GH_3 cells. Cells were grown to near confluence in standard medium and then transferred and maintained in medium containing 10% resin-treated FCS. The rT_3 was added to the culture medium before harvesting at the times indicated. I5'D activity was determined using a T_4 concentration of 50 nM and 20 mM DTT. Points represent the mean of triplicate determinations, which differed <5%.

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